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13. ABSTRACT *(Maximum 200 words)*

c-Myc is amplified in 16%, rearranged in 5%, and overexpressed in nearly 70% of all human breast cancers and it regulates both proliferation and apoptosis. Bcl-x_L, known to inhibit apoptosis potentially by modulating mitochondrial permeability and caspase activation, is overexpressed in some breast tumors and derivative cell lines. Pro-apoptotic Bax has been shown to be significantly reduced or altogether absent in many breast tumors and cell lines. Evidence from c-Myc/TGF-α bitransgenic mice suggests that escape from c-Myc-induced apoptosis may be necessary for continued cell cycle progression and neoplastic development.

The focus of this study is the resolution of the role of Bcl-x_L overexpression and Bax loss in cooperation with c-Myc overexpression in mammary tumorigenesis; constitutive expression of Bcl-x_L and loss of Bax likely disrupt the c-Myc-induced apoptotic pathways without significant alteration in c-Myc-mediated proliferation. c-Myc transgenic/Bax-knockout and c-Myc/Bcl-x_L bitransgenic mice have been generated, genotyped, and shall be assessed for altered tumor onset, incidence, growth, and pathological /molecular characteristics once mammary tumors arise. Utilization of these models will aid in the dissection of the role of apoptosis in the development of breast cancer.

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PI - Signature

15 September 1999
Date

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For Annual Report Grant # DAMD17-97-1-7110

"Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis"

P.I. Matthew Hunter Jamerson

This Annual Summary Report addresses Grant # DAMD17-97-1-7110, a Pre-Doctoral Training Fellowship, covering research conducted by the principal investigator Matthew Hunter Jamerson (an M.D./Ph.D. student at the Lombardi Cancer Center, Georgetown University Medical Center), entitled "Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis."

INTRODUCTION:

c-Myc is a 439 amino acid nuclear transcription factor that interacts with DNA when heterodimerized with the Max protein. This heterodimerization is required for c-Myc-mediated cell cycle progression, cell growth, cellular transformation, and apoptosis, and is facilitated via C-terminal leucine zipper and basic helix-loop-helix motifs (Harrington *et al.*, 1994; Packham *et al.*, 1995; Facchini *et al.*, 1998; Elend *et al.*, 1999). c-Myc plays a role in cellular transformation via transcription upregulation and repression of target genes. The former occurs through established E-box or other less well-defined promoter elements, while the latter is most likely mediated through initiator elements or in conjunction with other transcriptional modulators such as AP-2 and C/EBP (Facchini *et al.*, 1998; Dang, 1999). Deregulated expression of c-*myc*, via multiple mechanisms, including translocation, proviral insertion, gene amplification, point mutation, and direct transcriptional or translational effects, is a common feature of many human cancers (e.g. breast, lung, liver, and colon), and is thought to contribute to cellular proliferation and transformation when apoptosis is suppressed (Evan *et al.*, 1992; Santoni-Rugiu *et al.*, 1998; Dang, 1999). The Dual Signal model, as proposed by Gerard Evan, suggests that induction of apoptosis is an obligate function of c-myc expression and acts as a potent mechanism for suppression of tumorigenesis (Evan *et al.*, 1993). Interestingly, recent data has suggested that c-Myc may increase genomic instability and enhance tumorigenesis, as do mutator oncogenes like MSH1 and MLH1, without absolute requirement for continued c-Myc overexpression (Felsher *et al.*, 1999).

In human breast cancers, c-*myc* is amplified in approximately 16%, rearranged in roughly 5%, and overexpressed in the absence of gross locus alteration in nearly 70% of all cases, thus suggesting its importance in the genesis and/or progression of breast cancer (Nass *et al.*, 1997; Deming *et al.*, 1999). The role of c-*myc* expression in both normal mammary development and function as well as in mammary tumorigenesis is currently a burgeoning field of study. c-Myc expression is increased in the normal mammary gland during pregnancy-related proliferation, it is absent in differentiated mammary alveolar cells during lactation, and is again increased during the normal apoptotic mammary involution process (Strange *et al.*, 1992). c-Myc is believed to be a nuclear mediator of mitogenic signals incident upon cells from various receptor systems and is contributory to, but not sufficient for, mammary epithelial cell transformation (Leder *et al.*, 1986; Telang *et al.*, 1990). Constitutive expression of c-*myc* has been shown to partially transform both mouse and human mammary epithelial cells (MECs), such that they grow in soft agar in response to epidermal growth factor (EGF) and transforming growth factor α (TGFα) (anchorage-independent growth), and are no longer as dependent upon these factors for anchorage-dependent growth as are the parental, non-transformed cells (Telang *et al.*, 1990; Valverius *et al.*, 1990).

Three groups have independently developed transgenic mice that express the c-*myc* oncogene in a mammary-associated (MMTV-c-*myc*) or mammary-specific (WAP-c-*myc*) context (Stewart *et al.*, 1984; Schoenenberger *et al.*, 1988; Sandgren *et al.*, 1995). Another group has developed a mouse model, using a mammary tissue reconstitution method, in which the v-*myc* oncogene is expressed by a retrovirus throughout the reconstituted mammae (Edwards *et al.*,

1988). Both groups that generated WAP-c-*myc* transgenic mice reported an incidence of mammary tumors approaching 100% in multiparous animals, with all virgin animals remaining tumor-free over the observation period (to 14 months of age). These findings are as expected owing to the temporal window for the hormone-driven activity of the whey acid protein (WAP) gene promoter. The presence of mammary adenocarcinomas was reported as 100% for multiparous transgenic mice in which the murine mammary tumor virus long terminal repeat (MMTV-LTR) had been placed upstream of the mouse c-*myc* locus containing all three exons. Interestingly, WAP-c-*myc* and MMTV-c-*myc* female transgenic mice display lengthy tumor latencies and exquisite dependence upon pregnancy for tumor development, suggestive not only of the contribution but also of the insufficiency of c-*myc* in mammary tumorigenesis.

TGFα is a secreted, 50 amino acid glycoprotein, derived from an active, membrane-bound 160 amino acid precursor. TGFα demonstrates a high level of homology (~42%) with EGF (Martinez-Lacaci *et al.*, 1999), and both molecules bind the epidermal growth factor receptor (EGFR) with high affinity. TGFα binding to the EGFR (also termed c-ErbB1) has been demonstrated to result in receptor homodimerization as well as heterodimerization with c-ErbB2, c-ErbB3, and/or c-ErbB4, when these receptor family members are present. Receptor dimerization subsequently leads to autophosphorylation and activation of downstream signaling pathways including p42/44 MAPK, JNK/SAPK, PI3K, PLC, and camp/PKA (Dickson *et al.*, 1995; Siegel *et al.*, 1998; Martinez-Lacaci *et al.*, 1999). TGFα is expressed in the normal murine mammary gland within the basal cells of the epithelium and the terminal cells of the nascent end bud; it is also present in murine and human mammary glands during pregnancy and has been demonstrated to have similar growth promotional effects upon human and murine MECs in vitro (Salomon *et al.*, 1987; Valverius *et al.*, 1989; Bates *et al.*, 1990; Liscia *et al.*, 1990; Snedeker *et al.*, 1991; Martinez-Lacaci *et al.*, 1999). Early evidence demonstrated increased TGFα expression in mammary tumors versus normal mammary gland (Derynck *et al.*, 1987; Arteaga *et al.*, 1988; Bates *et al.*, 1988; Travers *et al.*, 1988); however, the current paradigm for EGF-family growth factor participation in breast cancer involves the establishment of a pro-proliferative, anti-apoptotic, autocrine/paracrine stimulatory loop with EGFR (a molecule found overexpressed in approximately 50% of human breast cancers) (Harris *et al.*, 1988; Dickson *et al.*, 1995; Dahiya *et al.*, 1998, DeLuca *et al.*, 1999). Three groups independently developed transgenic mouse models in which TGFα growth factor is expressed in a metal ion-inducible, general tissue context (MT-*tgfα*) (Sandgren *et al.*, 1990; Jhappan *et al.*, 1990), a mammary-associated context (MMTV-*tgfα*) (Matsui *et al.*, 1990), or a mammary-specific context (WAP-*tgfα*) (Sandgren *et al.*, 1995). Characterization of these transgenic models suggested that constitutive *tgfα* expression accelerates mammary development, impedes apoptotic involution, and contributes to mammary transformation by acting as both a survival and growth factor for differentiated murine MECs. Significantly, the requirement for pregnancy and extended tumor latency for *tgfα* transgenic models illustrates that TGFα is likely to be incapable of serving as the sole cause of mammary cancers; rather, it is likely to be one step along a multistep oncogenic pathway.

The MMTV-c-*myc*/MMTV-v-*Ha-ras* cross generated in 1987 was the first c-*myc* containing bitransgenic mouse (Sinn *et al.*, 1987). Characterization of this model demonstrated that deregulated expression these two genes resulted in accelerated mammary tumorigenesis with an abrogation of the requirement for pregnancy (mammary tumors were observed in virgin female mice as well as in male mice). In 1995, two c-*myc*/*tgfα* bitransgenic models were developed and lent support to the notion that signaling through the EGFR and/or activation of

Ras could potently synergize with deregulated c-*myc* expression in the mammary tumorigenic process (Amundadottir *et al.*, 1995; Sandgren *et al.*, 1995). The MMTV-c-*myc*/MT-*tgfα* bitransgenic mice developed multiple mammary adenocarcinomas with a much reduced latency and in the absence of any requirement for pregnancy or ovarian hormone stimulation. The complete absence of normal mammary tissue in bitransgenic animals and the ability of bitransgenic mammary tissue from 3 week-old mice to form tumors in athymic mice suggest that these two important, mammary-relevant genes are capable of synergistically transforming the mammary epithelium, apparently requiring minimal, if any, additional genetic alterations (Amundadottir *et al.*, 1995, 1996a). Characterization of the WAP-c-*myc*/WAP-*tgfα* bitransgenic model confirmed the potent synergy of these two genes in promoting and accelerating mammary tumor formation when compared with the relevant single transgenic animals (Sandgren *et al.*, 1995). Furthermore, the power of this cooperative interaction is demonstrated in both models since males and virgin female bitransgenic animals develop mammary tumors.

Subsequent work in our laboratory with single transgenic mice, c-*myc*/*tgfα* bitransgenic mice, and cell lines derived from transgenic mouse mammary tumors, has led to the hypothesis that TGFα can cooperate with c-Myc in promoting cell cycle progression and can act to suppress c-Myc-induced apoptosis (Amundadottir *et al.*, 1996b; Nass *et al.*, 1996, 1998). *In situ* end labeling of DNA fragments (TUNEL assay) in paraffin-embedded mammary tumor sections from transgenic animals indicate the presence of apoptotic mammary cells in c-*myc* transgenic tumors and their near absence in tumors from *tgfα* and c-*myc*/*tgfα* transgenic mice (Amundadottir *et al.*, 1996b). Data from tumor cell lines indicate that the overexpression of these two genes results in increased cell proliferation under both anchorage-dependent and –independent conditions, a reduced requirement for exogenous growth factor stimulation, and greatly decreased apoptosis. The cell lines derived from c-*myc* transgenic mouse mammary tumors were significantly more apoptotic than cell lines derived from *tgfα* and c-*myc*/*tgfα* transgenic mammary tumors; however, the frequency of apoptotic cells in the c-*myc* lines could be considerably suppressed by the addition of exogenous TGFα or EGF. Conversely, apoptosis was considerably accentuated when EGFR signaling was blocked by addition on PD153035, a specific, synthetic EGFR tyrosine kinase inhibitor (Amundadottir *et al.*, 1996b). Our results, together with those of another group, have suggested that transformation, maintenance of transformation, and suppression of apoptosis in c-*myc*-overexpressing mammary tumor cell lines derived from transgenic animals may require signaling through the p42/44 MAPK and PI3K pathways, both of which are targets of activated EGFR (Amundadottir *et al.*, 1998; Wang *et al.*, 1999).

Molecular characterization of apoptosis in c-*myc*-overexpressing murine MECs derived from the MMTV-c-*myc* transgenic mice led to the recognition that Bcl-x_L, an anti-apoptotic member of the Bcl-2 family of apoptosis regulatory proteins, is a likely mediator of TGFα and EGF-directed protection against c-*myc*-driven apoptosis. Bcl-x_L mRNA and protein levels were elevated with TGFα or EGF treatment of these c-*myc*-expressing cell lines and expression of this anti-apoptotic molecule was significantly diminished with growth factor removal, TGFβ treatment, or PD153035 treatment (EGFR blockade). Additionally, levels of Bax, a pro-apoptotic Bcl-2 family member, and p53 appeared relatively high and unchanged, while Bcl-2 and Bcl-x_S levels remained low or undetectable with these aforementioned treatments (Nass *et al.*, 1996). The work in our laboratory has led to the development of the following model explaining the cooperation between in c-Myc and TGFα in proliferation and apoptosis in the mouse mammary gland: First, deregulated c-Myc may drive cellular proliferation by

E2F1, cyclin E, cdc25A phosphatase, and CAK-activating partner cdk7, and by lowering p27 levels resulting in cdk2 activation. In contrast, TGFα overexpression leads to induction of cyclin D1 and, subsequently, the activation of cdk4/6 (Liao *et al.*, in press). The combination of these effects may further deregulate the cell cycle and abrogate normal cell cycle checkpoint controls. Second, deregulated c-*myc* expression may promote apoptosis by directly inducing p53 expression, and by directly or indirectly inducing Bax expression. Bax has been shown to be directly responsive to p53 and also to be a potential target for c-Myc induction owing to the location of four E-box motifs within the Bax promoter/5'-untranslated region (Miyashita *et al.*, 1995). This work, combined with results from the characterization of an MMTV-c-*myc*/WAP-*bcl2* bitransgenic model (*bcl2* expression accelerated mammary tumorigenesis and suppressed *in vivo* mammary tumor apoptosis) (Jäger *et al.*, 1997), strongly suggest that mammary tumorigenesis is significantly increased when deregulated c-*myc* expression, responsible both for driving proliferation and sensitizing cells to apoptosis, is coupled with other genetic alterations that act to block the c-*myc*-mediated apoptotic pathways.

Recently, a great deal of information has been published exploring the role of apoptosis regulatory proteins in the normal development of the mammary gland as well as in breast cancer. Bcl-x_L is expressed in the cuboidal epithelium and myoepithelium of the breast and is known to be decreased during post-lactational mammary gland involution with its splice variant, Bcl-x_S, being induced (Krajewski *et al.*, 1994a; Li *et al.*, 1997a). Whereas, Bcl-2 levels are reduced during the early stages of mammary involution, levels of Bcl-x_L and Bax are highly upregulated with the relative levels skewed toward greater pro-apoptotic protein expression (Schorr *et al.*, 1999). Bcl-x_L expression has been correlated with the presence of EGFR in ER-negative breast cancer cell lines with data from our lab regarding mammary tumor cell lines confirming that EGFR signal blockade results in decreased Bcl-x_L expression (Nass *et al.*, 1996; Hsu *et al.*, 1997). Bcl-x_L has been shown to block apoptosis induced by p53 in T47D and TNF / anti-Fas in MCF-7 breast cancer cell lines (Jäättelä *et al.*, 1995; Schott *et al.*, 1995). With regards to the *in vivo* situation, Bcl-x_L has been shown to be overexpressed in breast cancer as compared to adjacent, normal breast tissue with Bcl-x_L expression predominating over Bcl-2 expression in higher histological grade breast tumors with greater tumor cell resistance to apoptosis (Schott *et al.*, 1995; Ogretman *et al.*, 1996; Olopade *et al.*, 1997; Sierra *et al.*, 1998). To date, no work has been published on the targeting of a Bcl-x_L transgene to the mammary gland of transgenic mice.

Bax has been found to be expressed in the epithelium of the normal breast and has been demonstrated to be increased during post-lactational mammary gland involution without dependence upon functionally intact p53 protein (Krajewski *et al.*, 1994b; Li *et al.*, 1997b). The partial or total loss of Bax in knockout mice provided evidence that the presence of Bax was not necessary for mammary gland development and lactation (though a very small percentage of homozygous knockout animals did evidence some post-delivery lactational problems); furthermore, Bax nullizygous animals had reduced MEC apoptosis during the first stage of post-lactational mammary involution (Schorr *et al.*, 1999a; Schorr *et al.*, 1999b). Bax was found to be weakly expressed or absent in several breast cancer cell lines and transfection of Bax into breast cancer cell lines resulted in increased apoptotic sensitivity and diminished tumor proliferation in athymic mice (Bargou *et al.*, 1995; Bargou *et al.*, 1996). Bax was found to be highly expressed in normal breast tissue and absent (or nearly so) in invasive ductal breast tumors (Bargou *et al.*, 1995). Furthermore, significant reductions in Bax were found in 34% of primary breast tumors in women with metastatic breast cancer and expression was inversely

correlated with survival, treatment response, and metastasis (Krajewski *et al.*, 1995; Kapranos *et al.*, 1996).

Presently, only one study has been published in which the role of Bax loss has been correlated with mammary tumorigenesis (Shibata *et al.*, 1999). A transgenic mouse model for prostate cancer, in which the SV40 large T antigen (*TAg*) gene was placed under the control of the C3(1) prostatein gene regulatory elements, was also discovered to result in the ubiquitous development of mammary adenocarcinomas in all transgenic females (Maroulakou *et al.*, 1994). Subsequent investigation with this model led to the determination that apoptosis, as measured by TUNEL assay, was most pronounced in preneoplastic hyperplasias and associated with increased Bax expression, and was reduced in both normal MECs and mammary adenocarcinomas; furthermore, generation of crosses between the Tag mice and p53 nullizygous mice demonstrated that apoptosis was entirely independent of p53 status in the mammary gland and mammary tumors and that p53 status did not influence the expression of Bax (Shibata *et al.*, 1996; Shibata *et al.*, 1999). Characterization of C3(1)-*TAg*/*bax*-hemizygous and nullizygous mice demonstrated that partial loss of Bax resulted in reduced apoptosis in preneoplastic mammary lesions with subsequent increases in tumor growth rate, tumor number, and tumor mass. Interestingly, no alteration in apoptosis levels or cellular proliferation were discovered in mammary carcinomas in these animals and animals in which both alleles of Bax were eliminated evidenced a slightly reduced number of mammary lesions perhaps due to reduced mammary ductal formation in these animals (Shibata *et al.*, 1999). This study lends further weight to the notion that Bax is a tumor suppressor gene and is specifically relevant to tumorigenic processes in the mammary gland (Yin *et al.*, 1997; Shibata *et al.*, 1999).

Of great interest to those who study breast cancer and c-Myc is the nature of apoptosis signaling by c-Myc and its contribution to tumorigenesis. Constitutive expression of Bcl-x_L and/or loss of Bax are likely to disrupt the c-Myc-induced apoptotic pathways without significantly influencing c-Myc-mediated proliferation. Development of these combinatorial, mammary-relevant transgenic models (c-*myc*/*bcl-*x_L and c-*myc*/*bax*-knockout) will provide a convincing, *in vivo* method for dissecting the role of apoptosis in c-Myc-related mammary tumorigenesis and may provide greater resolution of molecular pathways that might be exploited for clinical assessment and therapeutic management of breast cancer.

SUMMARY OF TRAINING AND RESEARCH ACCOMPLISHMENTS:

This annual summary of training and research accomplishments covers the period between August 1, 1998 and December 31, 1999 for Grant #DAMD17-97-1-7110 under the direction of principal investigator, Matthew Hunter Jamerson. Please note that a revised statement of work will be presented within this summary discussion.

I. Revised Specific Aim #1: Develop two transgenic model systems to examine the cooperation of c-Myc with Bax-knockout and Bcl-x_L expression in mammary tumorigenesis. *Months 1-22*

A. Specific Aim #1A: Generate tetTA(tetOP-*tta*)tetOP-*luc*/tetOP-*bcl-x*$_L$/MMTV-c-*myc* transgenic mice, ascertain transgene expression using tail DNA biopsy, and establish study groups.

B. Specific Aim #1B: Generate MMTV-c-*myc*/Bax-knockout transgenic mice, ascertain transgene expression (or lack thereof) using tail DNA biopsy, and establish study groups.

Training and Research Accomplishments for Specific Aim #1A:

Two MMTV-c-*myc* males in the FVB background were obtained from the Charles River Laboratories (Wilmington, MA) in September 1998 and were used to develop a breeding colony of c-Myc transgenic mice through matings with normal FVB female mice under a current breeding license with DuPont Medical Products (Wilmington, DE). This particular breeding strategy is dictated by the fact that c-Myc female mice are nearly always incapable of nursing their young and subsequently the pups succumb to starvation or cannabalism. Ascertainment of the transgenic status of c-Myc mice is conducted using a convenient PCR-based strategy. Genomic DNA is obtained from Proteinase K /SDS-based digestion and phenol/chloroform extraction of tail biopsy material and is utilized in a PCR reaction with two MMTV-c-*myc* transgene-specific primers MMTV-Myc5' primer as [5'-CCC AAG GCT TAA GTA AGT TTT TGG-3'] and MMTV-Myc3' primer as [5'-GGG CAT AAG CAC AGA TAA AAC ACT-3'] and PCR Supermix. Transgene positive animals are identified by the resolution of a single band of approximately 880bp on a 1.0% agarose gel (**Figure 1**).

Four breeding pairs of tetOP-*bcl-x*$_L$ transgenic mice were obtained from the laboratory of Dr. Priscilla A. Furth (University of Maryland, Baltimore, MD) in April 1998 and were subsequently used to develop a breeding colony of Bcl-x_L transgenic mice. Ascertainment of the transgenic status of Bcl-x_L mice is conducted using a PCR-based strategy. The primers used in this reaction are BCLTG5 primer as [5'-GCA TTC AGT GAC CTG ACA TC-3'] and BCLTG3 primer as [5'-CTG AAG AGT GAG CCC AGC AGA ACC-3'] and transgene positive animals are identified by the resolution of a single band of approximately 450bp (**Figure 2**).

In the absence of reliability confirmation and with the delayed availability of the MMTV-*tta* transgenic mice, we have chosen to use the tetTA(tetOP-*tta*)tetOP-*luc* transgenic mouse for control of the tetOP-*bcl-x*$_L$ transgene. This transgenic mouse was developed as a self-inducing tetracycline-regulable system wherein the tetracycline transactivator protein (tTA) gene and the luciferase (*luc*) gene are expressed under the control of a minimal human cytomegalovirus (hCMV) promoter and a series of tandemly-repeated tetracycline responsive operons (tetOP) (Shockett *et al.*, 1995). It should be noted that this system is a tet-OFF system; therefore, in the absence of the antibiotic tetracycline (or derivative doxycycline), constitutive expression of the

tTA drives the expression of transgenes possessing the tetOP elements. Two breeding pairs of tetTA(tetOP-*tta*)tetOP-*luc* transgenic mice were obtained from the Jackson Laboratories (Bar Harbor, ME) in July 1998 and were subsequently used to develop a breeding colony of these animals. Ascertainment of the transgenic status of tTA mice is conducted using a PCR-based strategy. The primers used in this reaction are CMVF1 primer as [5'-TGA CCT CCA TAG AAG ACA CC-3'] and TTAREV1 primer as [5'-ATC TCA ATG GCT AAG GCG TC-3'] and transgene positive animals are identified by the resolution of a single band of approximately 290bp (**Figure 3**).

Following establishment of breeding colonies for each of these transgenic mice, tetOP-*bcl-x$_L$* transgenic mice were mated with tetTA(tetOP-*tta*)tetOP-*luc* resulting in animals in which the expression of Bcl-x$_L$ should be constitutively present in nearly all tissues in the mice. Confirmation of Bcl-x$_L$ expression was achieved by Western blot analysis on a 10% reducing polyacrylamide gel using a Transduction Laboratories rabbit anti-human/mouse Bcl-x$_L$ primary antibody, an HRP-conjugated New England Biolabs goat anti-rabbit secondary antibody, and ECL Super Signal reagent from Pierce (**Figure 4**). Finally, female tetTA(tetOP-*tta*)tetOP-*luc*/tetOP-*bcl-x$_L$* bitransgenic mice were mated with male MMTV-c-*myc* transgenic mice to yield an F$_1$ study population. For tumor studies, females will be recruited into one of three groups: the virgin tumor group (15 mice per major genotype/5 mice per minor), the parous tumor group (10 mice per major genotype), or the developmental group (2-3 mice per time period). The current status of transgenic animal generation and study group recruitment for the c-Myc/Bcl-x$_L$ cross is represented in **Table 1**.

Training and Research Accomplishments for Specific Aim #1B:

Two male and four female Bax-knockout mice were obtained from the laboratory of Dr. Priscilla A. Furth (University of Maryland, Baltimore, MD) in March 1998 and were subsequently used to develop a breeding colony of Bax-hemizygous and -nullizygous animals. Ascertainment of the transgenic status of Bax-knockout mice is conducted using a three primer, PCR-based strategy. The primers used in this reaction are BPR2 primer as [5'-GTT GAC CAG AGT GGC GTA GG-3'], MK1 primer as [5'-GAG CTG ATC AGA ACC ATC ATG-3'], and NPR2 primer as [5'-CCG CTT CCA TTG CTC AGC GG-3']. Bax wild-type animals are identified by the presence of a single band of approximately 320bp, Bax-nullizygous animals are identified by the presence of a single band of approximately 600bp, and Bax-hemizygous animals are identified by the presence of both bands (indicative of a wild-type Bax allele and a disrupted Bax allele) (**Figure 5**).

Since Bax-nullizygous males are infertile due a blockade of the spermatogenic process and an accumulation of premeiotic germ cells and therefore are not useful as breeders (Knudson *et al.*, 1995) and these knockout mice are based on a C57B6/albino strain mixture, a two generation breeding strategy was employed to generate transgenic animals possessing c-Myc in the presence/absence of Bax. First, MMTV-c-*myc* males were mated with Bax-nullizygous females to generate F$_1$ generation MMTV-c-*myc*/Bax-hemizygous breeder males. Subsequently, these F$_1$ breeder males were mated to Bax-nullizygous females to generate mice of all possible genotypes yielding an F$_2$ study population. For tumor studies, females will be recruited into one of three groups: the virgin tumor group (15 mice per major genotype/5 mice per minor), the parous tumor group (10 mice per major genotype), or the developmental group (2-3 mice per time period). The current status of transgenic animal generation and study group recruitment for the c-Myc/Bax-knockout cross is represented in **Table 2**.

II. Revised Specific Aim #2: Evaluate alterations in mammary tumorigenesis and mammary development resulting from the cooperation of c-Myc and Bcl-x_L and c-Myc and Bax-knockout. *Months 22-36*

A. Specific Aim #2A: Follow F_1 generation study animals (c-Myc/tTA/Bcl-x_L cross) for tumor latency, incidence, multiplicity, growth kinetics, metastasis, and dependence upon parity.

B. Specific Aim #2B: Follow F_2 generation study animals (c-Myc/Bax-knockout cross) for tumor latency, incidence, multiplicity, growth kinetics, metastasis, and dependence upon parity.

C. Specific Aim #2C: Assess whether deregulated expression of c-Myc coupled with constitutive expression of Bcl-x_L or loss of Bax results in abnormal mammary gland development.

Training, Research Accomplishments, and Future Directions for Specific Aim #2A:

The virgin tumor group of study animals will be followed until they develop mammary tumors or succumb to another illness. Animals will be examined three times a week for the development of mammary tumors and shall be sacrificed prior to tumor growth to 10% of animal body weight. Tumor onset and growth, as measure by calipers, will be determined starting from day 1 at birth and will be compared across all major (tTA/wt/wt, tTA/x_L/wt, tTA/wt/myc, tTA/x_L/myc) and minor (wt/wt/wt, wt/x_L/wt, wt/wt/myc, wt/x_L/myc) genotypes. Presence of metastasis will be determined initially by gross observation of organs at time of death or sacrifice and will be further assessed by serial sectioning of paraformaldehyde-fixed, paraffin-embedded, harvested organs (lungs, liver, spleen).

The parous tumor group of study animals will be used to determine the influence of pregnancy and ovarian hormone stimulation on mammary tumor development. A total of ten animals of each major genotype (tTA/wt/wt, tTA/x_L/wt, tTA/wt/myc, tTA/x_L/myc) will be repetitively mated with FVB males starting at the age of 10 weeks and will be followed until they develop mammary tumors or succumb to another illness. All offspring of these parous females will be removed following either twenty-days of whelping or death of the entire litter. Examinations and measurements will be conducted in the aforementioned manner.

Training, Research Accomplishments, and Future Directions for Specific Aim #2B:

All examinations and measurements will be conducted as described in Specific Aim #2A with the major genotypes comprising (myc $bax^{-/-}$, myc $bax^{+/-}$, myc $bax^{+/+}$, and wt $bax^{-/-}$) and the minor genotypes comprising (wt $bax^{+/-}$ and wt $bax^{+/+}$).

Training, Research Accomplishments, and Future Directions for Specific Aim #2C:

The overall development of the mammary gland predominately occurs during the post-natal period with the exception of the formation of the mammary rudiment (anlage). Prior to the onset of puberty, mammary ductal expansion remains minimal; whereas, at the onset of puberty (approximately 4-5 weeks of age in mice), mammary ducts began to extend well into the mammary fat pads and branch out with the formation of highly proliferative terminal end bud

structures. At 10-12 weeks of age, the terminal end bud structures disappear and the majority of the mammary fat pad is filled with ducts. It is not until pregnancy and parturition occur that complete alveolar formation and mammary epithelial cell terminal differentiation is achieved (Robinson *et al.*, 1995; Hennighausen *et al.*, 1998).

Two to three female mice of each major genotype for both the c-Myc/tTA/Bcl-x_L and c-Myc/Bax-knockout studies will be utilized to study the influence of these transgenes on mammary development. Mice will be sacrificed by CO_2 asphyxiation, mammary gland whole mounts will be prepared as per Amundadottir *et al.* (1995), and the remaining mammary tissue will be fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for histological and immunohistochemical analyses as described in Specific Aim #3. Mice for these development studies will be sacrificed at the following ages: 3, 6, 9, and 12 weeks, pregnancy day 7, pregnancy day 14, lactation day 1, lactation day 10, involution day 1, involution day 3, and involution day 10 (involution will be promoted by removal of pups at day 10 of lactation).

III. Revised Specific Aim #3: Evaluate transgene expression (or lack thereof), apoptosis and proliferation indices, and histology from transgenic animal tumors and normal mammary tissue. *Months 34-42*

Training, Research Accomplishments, and Future Directions for Specific Aim #3:

Histological analysis will be conducted on 4% paraformaldehyde-fixed, paraffin-embedded, hematoxylin/eosin-stained mammary tumor and normal mammary gland tissues. Apoptotic indices for tumor, hyperplastic, and normal tissue will be evaluated using the terminal deoxynucleotide transferase-mediated digoxigenin-dUTP nick end labeling (TUNEL) method on paraformaldehyde-fixed, paraffin-embedded sections as per Trevigen (Liao *et al.*, in press). Proliferation indices for tumor, hyperplastic, and normal tissue will be evaluated using PCNA immunohistochemistry with a mouse anti-PCNA antibody from Dako on paraformaldehyde-fixed, paraffin-embedded sections as per Shibata *et al.* (1999).

Evaluation of transgene expression (or lack thereof) will be conducted using any and/or all of the following: immunohistochemistry on paraformaldehyde-fixed, paraffin-embedded tissue sections, *in situ* hybridization assay on paraformaldehyde-fixed, paraffin-embedded tissue sections, and Western blot analysis on mammary gland whole-cell lysates using cytokeratin 8 as a marker for mammary gland epithelial content (Amundadottir *et al.*, 1995; Shibata *et al.*, 1999; Rauh *et al.*, 1999; Liao *et al.*, in press) Antibodies for Western blot analysis are as follows: rabbit anti-human/mouse Bax (N-20), rabbit anti-human/mouse Bcl-$x_{L/S}$ (S-18), and rabbit anti-human/mouse c-Myc (C-19) from Santa Cruz. Antibodies for immunohistochemistry are as follows: rabbit anti-human/mouse Bax (N-20), rabbit anti-human/mouse Bcl-$x_{L/S}$ (S-18), and rabbit anti-human/mouse c-Myc (C-19) from Santa Cruz .

IV. Revised Specific Aim #4: Evaluate the growth factor dependence, growth inhibitor independence, apoptosis resistance, and tumorigenicity of cell lines derived from transgenic murine mammary tumors and mammary tissues. *Months 42-48*

Training, Research Accomplishments, and Future Directions for Specific Aim #4:

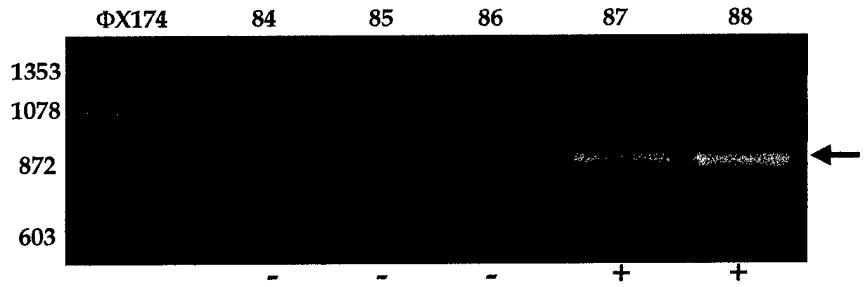
Cell lines will be developed from tumors and/or from non-tumorous mammary tissue from the transgenic study animals as per Amundadottir *et al.* (1995). Cell lines will be evaluated *in vitro* for proliferation and apoptotic end-points as determined by *in vitro* culture conditions and treatments with growth factors (TGFα, EGF, bFGF, IGF1), growth inhibitors (TGFβ), and apoptosis-inducing treatments (TNF, anti-Fas). The tumorigenicity of these developed cell lines will then be assessed by subcutaneous injection of each cell line into female, athymic mice; all surgical procedures will be performed with recipient animals under metofane anesthesia.

CONCLUSIONS:

During this second year of work on Grant #DAMD17-97-1-7110, the significant goal of generation of the relevant transgenic murine mammary tumor models has been achieved despite numerous initial setbacks involving animal availability, animal fecundity/sterility, and breeding scheme complexity. As presented in **Figures 1** and **2**, the majority of the necessary animals have been recruited to the virgin and parous study groups and the remainder of these animals should be generated in the course of the next few months. Work is also currently underway to establish whole-mount and immunohistochemical procedures for use on the established aims of this project. Additionally significant have been the numerous research-oriented skills that this principal investigator has acquired during the course of work on this grant, to include the following: mouse breeding and husbandry, animal surgical procedures, mouse embryo harvesting and DNA microinjection, transgenic mouse generation, PCR, cell and tissue culture, Western blot analysis, mammary gland whole mount preparation, tissue fixation and preparation, and immunohistochemistry.

FIGURE 1

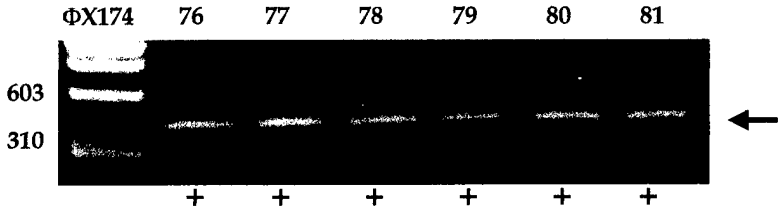
PCR Assessment of Mouse Genotype: c-Myc



Template: Mouse Tail DNA - P/C extraction
Primers: myc2 = 5′-ggg cat aag cac aga taa aac act-3′
myc1 = 5′-ccc aag gct taa gta agt ttt tgg-3′
PCR: 95° 1:00 / 52° 1:00 / 72° 1:15 x 42 cycles

FIGURE 2

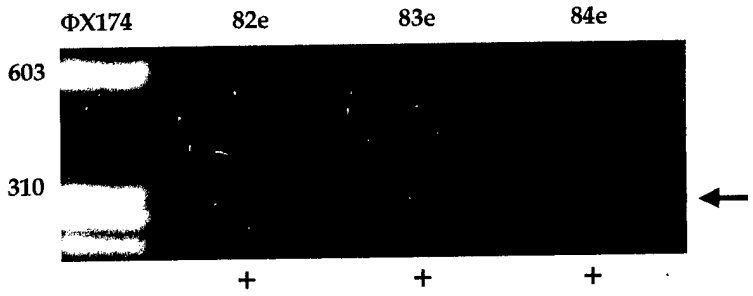
PCR Assessment of Mouse Genotype: Bcl-x_L



Template: Mouse Tail DNA - P/C extraction
Primers: bcltg3 = 5′-ctg aag agt gag ccc agc aga acc-3′
bcltg5 = 5′-gca ttc agt gac ctg aca tc-3′
PCR: 95° 1:00 / 58° 1:00 / 72° 3:00 x 30 cycles

FIGURE 3

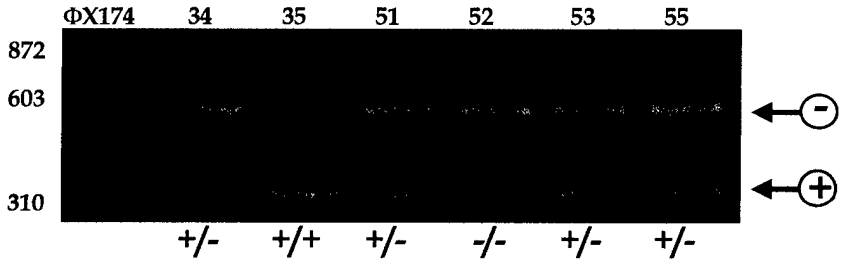
PCR Assessment of Mouse Genotype: tTA-Luc



Template: Mouse Tail DNA - P/C extraction
Primers: cmvf1 = 5′-tga cct cca tag aag aca cc-3′
ttarev1 = 5′-atc tca atg gct aag gcg tc-3′
PCR: 94° 0:45 / 53° 0:45 / 72° 1:30 x 30 cycles

FIGURE 4

PCR Assessment of Mouse Genotype: Bax

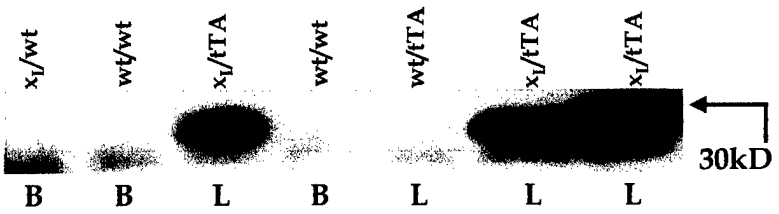


Template: Mouse Tail DNA - P/C extraction
Primers: bpr2 = 5′-gtt gac cag agt ggc gta gg-3′
mk1 = 5′-gag ctg atc aga acc atc atg-3′
npr2 = 5′-ccg ctt cca ttg ctc agc gg-3′
PCR: 94° 0:45 / 55° 1:30 / 72° 2:00 x 35 cycles

FIGURE 5

Directional Breeding Strategies: Confirmation of Tet System Activity #1

- tTA-Luc Mouse = 7 tetOP + ^CMV Pr. + tet Transact.
  - tetOP-Luciferase
  - system ON in absence of tetracycline
- tetOP-Bcl-x_L Mouse = 7 tetOP + ^CMV Pr. + hBcl-x_L



- All virgin females without tetracycline treatment

TABLE 1

MAJOR GENOTYPES / Virgin Group [15 mice per]															
tTA/xL/myc	95h	10i	12i	13i	9l	99l	18m	22m	34n	37n	46q	47q	21s	8t	19t
Age (d)	*236*	*235*	*234*	*234*	*168*	*148*	*148*	*144*	*125*	*125*	*77*	*77*	*47*	*36*	*35*
tTA/xL/wt	99h	1i	14i	50i	58i	55j	63k	65k	19l	89m	63o	64o	13r	18r	19r
Age (d)	*236*	*236*	*234*	*219*	*219*	*206*	*179*	*179*	*168*	*136*	*103*	*103*	*62*	*60*	*60*
tTA/wt/myc	11i	52i	57j	66k	15m	58o	20p	23p	33p	58p	62p	62r	64r	20s	25t
Age (d)	*235*	*219*	*206*	*179*	*148*	*105*	*90*	*90*	*90*	*83*	*83*	*49*	*49*	*47*	*35*
tTA/wt/wt	84h	97h	7i	8i	51i	50j	53j	74j	86j	98j	15l	29m	8n	76o	80o
Age (d)	*236*	*236*	*235*	*235*	*219*	*206*	*206*	*190*	*190*	*190*	*168*	*144*	*133*	*100*	*100*
MINOR GENOTYPES / Virgin Group [5 mice per]															
wt/xL/myc	18i	47i	64j	79j	19p										
Age (d)	*232*	*219*	*190*	*188*	*90*										
wt/xL/wt	3i	6i	48i	53i	60p										
Age (d)	*236*	*236*	*219*	*219*	*83*										
wt/wt/myc	88h	9i	69k	71k	10l										
Age (d)	*236*	*235*	*179*	*179*	*168*										
wt/wt/wt	94h	96h	2i	56i	25p										
Age (d)	*236*	*236*	*236*	*219*	*90*										
MAJOR GENOTYPES / Parous Group [10 mice per]															
tTA/xL/myc	62t														
Age (d)	*26*														
tTA/xL/wt	33s	36u													
Age (d)	*42*	*8*													
tTA/wt/myc															
Age (d)															
tTA/wt/wt	***52q***	***16r***	10t	11t	16t	30t	34t	60t							
Age (d)	*77*	*58*	*35*	*35*	*35*	*34*	*32*	*26*							

TABLE 2

MAJOR GENOTYPES / Virgin Group [15 mice per]															
myc bax -/-	1j	11j	12j	20j	21j	29j	37k	38k	39k	97k	43l	65l	64m	89n	10o
Age (d)	*212*	*205*	*205*	*205*	*205*	*205*	*176*	*176*	*176*	*165*	*156*	*151*	*136*	*114*	*114*
myc bax +/-	24i	69i	82i	84i	93i	94i	2j	19j	35j	36j	39j	61j	6k	43k	47k
Age (d)	*222*	*212*	*212*	*212*	*212*	*212*	*212*	*205*	*205*	*205*	*205*	*193*	*184*	*176*	*176*
myc bax +/+	15j	34j	37j	38j	83k	4l	58m	55n	73n	81n	34o	35o	90o	12p	92p
Age (d)	*205*	*205*	*205*	*205*	*165*	*165*	*136*	*122*	*119*	*115*	*115*	*115*	*92*	*91*	*77*
wt bax -/-	89i	90i	91i	31j	81k	27l	44l	11p	42r	51r	58r	60s	61s	81s	94s
Age (d)	*212*	*212*	*212*	*205*	*165*	*162*	*156*	*91*	*51*	*51*	*45*	*35*	*35*	*32*	*32*
MINOR GENOTYPES / Virgin Group [5 mice per]															
wt bax +/-	23i	28i	30i	5j	47p										
Age (d)	*222*	*222*	*222*	*212*	*83*										
wt bax +/+	27i	31i	77i	96i	24j										
Age (d)	*222*	*222*	*212*	*212*	*205*										
MAJOR GENOTYPES / Parous Group [10 mice per]															
myc bax -/-	***45n***	***96o***	***71p***	***72p***	***81p***	***88p***	27r	43r	54r	81r					
Age (d)	*122*	*92*	*79*	*79*	*78*	*77*	*53*	*51*	*45*	*44*					
myc bax +/-	***47n***	***54n***	***76n***	***82n***	***73p***	***79p***	***83p***	***90p***	***22q***	***65q***					
Age (d)	*122*	*122*	*118*	*115*	*79*	*79*	*78*	*77*	*74*	*69*					
myc bax +/+	***21q***	***67q***	52r	53r	4s	43s	46s	64s	83s	97s					
Age (d)	*74*	*69*	*51*	*51*	*43*	*37*	*36*	*35*	*32*	*32*					
wt bax -/-	22u	23u													
Age (d)	*7*	*7*													

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APPENDIX A

KEY RESEARCH ACCOMPLISHMENTS for GRANT DAMD17-97-1-7110:

- Development of effective breeding strategies for the generation of the two transgenic models (c-Myc/tTA/Bcl-x_L and c-Myc/Bax-knockout)

- Optimization of PCR-based assays for c-Myc, Bcl-x_L, and tTA transgenic and Bax-knockout mouse genotyping

- Confirmation of Bcl-x_L expression in mammary gland whole cell lysates in tetOP-*tta*/tetOP-*bcl-x_L* bitransgenic mice

- Establishment of mammary gland whole-mount procedure for assessment of transgene-induced alterations in mammary gland development

- Recruitment of all virgin tumor group animals, both major and minor genotypes, for both transgenic murine mammary tumor models

- Recruitment of 80% of all parous tumor group animals for the c-Myc/Bax-knockout transgenic model (approximately 45% have reached 10 weeks of age)

- Recruitment of nearly 28% of all parous tumor group animals for the c-Myc/tTA/Bcl-x_L transgenic model (approximately 5% have reached 10 weeks of age)

APPENDIX B

REPORTABLE OUTCOMES LIST for GRANT DAMD17-97-1-7110:

Accepted Manuscript:

1. Jamerson MH, Johnson MD and Dickson RB. Dual Regulation of Proliferation and Apoptosis: c-*Myc* in Bitransgenic Murine Mammary Tumor Models. Oncogene. "paper in press."

☐ Copy of Galley Proof Attached following this page

Abstract Presentation:

1. Jamerson MH, Johnson MD and Dickson RB. Cooperation of c-Myc, Bcl-x_L, and Bax-Knockout in Mammary Tumorigenesis. Lombardi Cancer Center Research Days. February 1999.

☐ Copy of Abstract Attached following this page

Informatics – Animals Models:

1. Generated triple transgenic murine model: tetTA(tetOP-*tta*)tetOP-*luc* / tetOP-*bcl-x*$_L$ / MMTV-c-*myc*

2. Generated transgenic / knockout murine model: MMTV-c-*myc* / Bax-Knockout

APPENDIX C:

Abstract #1: Jamerson MH, Johnson MD and Dickson RB. Cooperation of c-Myc, Bcl-x_L, and Bax-Knockout in Mammary Tumorigenesis. Lombardi Cancer Center Research Days. February 1999.

c-Myc oncogene has been reported to be amplified in 25-30% of human breast cancers and overexpressed in more than 70% of human breast cancers. Analysis *in vitro* has demonstrated that c-Myc is involved in signaling for cell proliferation and apoptosis. The Bcl-x_L protein, an anti-apoptotic member of the Bcl-2 apoptosis-modulatory protein family, is known to block apoptotic cell death under a wide variety of conditions and has been shown to be overexpressed in some human breast cancers and breast cancer cell lines. The Bax protein, a pro-apoptotic member of the Bcl-2 protein family, is known to contribute to cellular vulnerability to apoptosis, has been demonstrated to possess a tumor suppressor-like function in human tumors, and has been shown to be weakly expressed or absent in human breast cancers and breast cancer cell lines.

Evidence from a c-Myc/TGFα bitransgenic mouse model suggests that escape from c-Myc-induced apoptosis may be necessary for continued cell cycle progression and neoplastic development. The focus of these studies is to determine if there is a synergism between deregulated c-Myc expression and loss/diminution of apoptosis in mouse mammary tumorigenesis. We hypothesize that the constitutive expression of c-Myc and Bcl-x_L will facilitate mammary tumorigenesis as a result of Bcl-x_L blockade of c-Myc-induced apoptosis and not c-Myc-mediated cell cycle progression. It is further predicted that the constitutive expression of c-Myc in a Bax-null background will also facilitate mammary tumorigenesis due to a disruption of the c-Myc-induced apoptotic pathways.

Work to date in support of this project includes the following: the establishment and optimization of PCR-based procedures for the identification of mouse transgenic status, the establishment of breeding colonies of c-Myc, tTA-Luc, and tetOP-Bcl-x_L transgenic animals and Bax-knockout animals, the evaluation and solution of breeding and nursing problems, and the establishment of a breeding program to achieve sufficient numbers of bitransgenic and control animals for study. Recent progress and current work is focused on the generation of additional strategies for these breeding experiments and involves the production of another Bcl-x_L transgenic mouse model without tetracycline regulatory elements. Furthermore, we are pursuing the creation of c-Myc and Bcl-x_L retroviruses for use in the establishment of bitransgenic mammary glands.

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Dual regulation of proliferation and apoptosis: c-*myc* in bitransgenic murine mammary tumor models

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Recent progress in the study of c-Myc has convincingly demonstrated that it possesses a dual role in regulating both proliferation and apoptosis; however, the manner in which c-Myc influences these cellular response pathways remains incompletely characterized. Deregulation of c-Myc expression, via multiple mechanisms, is a common feature of multiple cancers and is an especially prominent feature of many breast cancers. Of significant interest to those who study mammary gland development and neoplasia is the unresolved nature and contribution of apoptosis to breast tumorigenesis. Recently, the use of transgenic mice and gene-knockout mice has allowed investigators to evaluate the pathological mechanisms by which different genes influence tumor development and progression. In this review, we address two distinct c-*myc*-containing bitransgenic murine mammary tumor models and discuss the contribution and possible future directions for resolution of cancer-relevant molecular pathways influenced by c-Myc.

Keywords: transgenic mice; mammary gland; c-*myc*; TGFα; *p53*

Introduction

The use of transgenic mice and mice bearing targeted gene disruptions (knockout mice) has given rise to current paradigms for the mechanistic evaluation of processes relevant to both physiology (e.g., embryogenesis, growth control and differentiation, morphogenesis) and pathology (e.g., neurodegenerative disease, hypertension, rheumatoid arthritis, neoplasia). Over 20 years ago, the combination of murine embryo culture with the techniques of reimplantation, DNA microinjection, and mammalian retrovirus manipulation resulted in the generation of the first transgenic mice. These animals were produced by embryo infection and microinjection methodologies (Brinster, 1972; Jaenisch, 1976; Gordon *et al*., 1980). Three years later, the first example of a tissue-specific transgenic animal was published (Igκ gene expression in murine spleen), thus establishing the refined capacity for examining exogenous gene expression in models with greater *in vivo* relevance (Brinster *et al*., 1983). Then, in 1984, the first transgenic animal was generated for the purpose of evaluating the relevance of a cellular proto-oncogene, c-*myc*, to mammary development and tumorigenesis (Stewart *et al*., 1984). Subsequently, a burgeoning field of mammary-specific transgenic murine models has been generated and characterized, greatly advancing our understanding of the molecular basis for the contribution of growth factors, oncogenes and tumor suppressor genes to the pathogenesis of breast cancer.

In this review, we will address two different c-*myc*-containing bitransgenic murine models (c-*myc*/*tgfα* and c-*myc*/*p53*$^{-/-}$) that our group (Amundadottir *et al*., 1995; McCormack *et al*., 1998) and two other groups (Elson *et al*., 1995; Sandgren *et al*., 1995) have generated. We shall also discuss the contributions these models have made to our understanding of breast cancer and of molecular pathways that are influenced by the c-*myc* oncogene.

c-myc *oncogene, the mammae and breast cancer*

c-Myc is a 439-amino acid nuclear transcription factor that interacts with DNA when heterodimerized with the Max protein. This heterodimerization is required for c-Myc-mediated cell cycle progression, transformation, and apoptosis, and is facilitated via C-terminal leucine zipper and basic helix-loop-helix motifs (Harrington *et al*., 1994; Packham *et al*., 1995). c-Myc has been demonstrated to contribute to a number of important cellular functions, including cell cycle progression, apoptosis and DNA anabolism. In addition, c-Myc plays a role in cellular transformation via both transcriptional upregulation and transcriptional repression of target genes. The former occurs through established E-box or other less well-defined promoter elements, while the latter is most likely mediated through initiator elements or in conjunction with other transcriptional modulators such as AP-2 and C/EBP (Facchini *et al*., 1998; Dang, 1999). The Dual Signal model, as proposed by Gerard Evan, suggests that induction of apoptosis is an obligate function of c-*myc* expression and acts as a potent mechanism for the suppression of tumorigenesis (Evan *et al*., 1993). c-Myc expression, coupled with any block to cellular proliferation, such as growth arrest caused by serum or growth factor deprivation, has been demonstrated in fibroblasts to result in apoptosis, independent of cell cycle phase (Evan *et al*., 1992). However, this does not occur in benzo[a]pyrene-immortalized human mammary epithelial cells (MECs) transfected with c-*myc* and deprived of epidermal growth factor (EGF) (Nass *et al*., 1998). Instead, these cells arrest in the G1 phase of the cell cycle and do not undergo apoptosis.

c-Myc expression is increased in the normal mammary gland during pregnancy-related prolifera-

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tion, it is absent in differentiated mammary alveolar cells during lactation, and it is again increased during the normal apoptotic mammary involution process (Strange *et al.*, 1992). c-Myc is believed to be a nuclear mediator of mitogenic signals incident upon cells from various receptor systems and is contributory to, but not sufficient for, mammary epithelial cell transformation (Leder *et al.*, 1986; Telang *et al.*, 1990). Constitutive expression of c-*myc* has been shown to partially transform both mouse and human MECs, such that they grow in soft agar in response to EGF and transforming growth factor α (TGFα) (anchorage-independent growth), and are no longer as dependent upon these growth factors for anchorage-dependent growth as are the parental, non-transfected cells (Telang *et al.*, 1990; Valverius *et al.*, 1990). Furthermore, deregulated expression of c-*myc*, via multiple mechanisms, including translocation, proviral insertion, gene amplification, point mutation, and direct transcriptional effects, is a common feature of many human cancers (including breast, lung, liver and colon), and is thought to contribute to cellular proliferation and transformation when apoptosis is suppressed (Evan *et al.*, 1992; Santoni-Rugiu *et al.*, 1998; Dang, 1999). In human breast cancers, c-*myc* is amplified in approximately 16%, rearranged in approximately 5%, and overexpressed in the absence of gross locus alteration in nearly 70% of all cases, thus suggesting its importance in the genesis and/or progression of breast cancer (Nass *et al.*, 1997; Deming *et al.*, 1999).

Three groups have independently developed transgenic mice that express the c-*myc* oncogene in a mammary-associated (MMTV-c-*myc*) or mammary-specific (WAP-c-*myc*) context (Stewart *et al.*, 1984; Schoenenberger *et al.*, 1988; Sandgren *et al.*, 1995). In addition to these c-*myc* transgenic animals, another group has developed a mouse model, using a mammary tissue reconstitution method, in which the v-*myc* oncogene is expressed by a retrovirus throughout the reconstituted mammae (Edwards *et al.*, 1988). Both groups that have generated WAP-c-*myc* transgenic mice have reported a high incidence of mammary tumors; Schoenenberger described the tumors as adenocarcinomas, while Sandgren described them as solid carcinomas. In both cases, tumor incidence approached 100% in multiparous animals, with all virgin animals remaining tumor-free over the observation period (to 14 months of age). Additionally, both groups reported the expression of the c-*myc* transgene in both neoplastic mammary tissue as well as in mammary tissue from normal female mice during the latter part of pregnancy and throughout lactation (Schoenenberger *et al.*, 1988; Sandgren *et al.*, 1995). These findings are as expected owing to the temporal window for the hormone-driven activity of the whey acid protein (WAP) gene promoter. Stewart *et al.*, (1984) reported the presence of mammary adenocarcinomas in 100% of multiparous F1 female transgenic mice derived from founder 141-3 in which the murine mammary tumor virus long terminal repeat (MMTV-LTR) had been placed immediately upstream of the mouse c-*myc* locus containing all three exons. Interestingly, WAP-c-*myc* and MMTV-c-*myc* female transgenic mice display lengthy tumor latencies and exquisite dependence upon pregnancy for tumor development, suggestive not only of the contribution but also of the insufficiency of c-*myc* in mammary tumorigenesis.

Transforming growth factor α, the mammae and breast cancer

TGFα is a secreted, 50-amino acid glycoprotein, derived from an active, membrane-bound 160-amino acid precursor. TGFα demonstrates a high level of homology (~42%) with EGF (Martinez-Lacaci *et al.*, 1999), and both molecules bind the epidermal growth factor receptor (EGFR) with high affinity. The growth factor family to which TGFα and EGF belong is now known to contain about 15 mammalian genes (Martinez-Lacaci *et al.*, 1999). TGFα binding to EGFR (also termed c-ErbB1) has been demonstrated to result in receptor homodimerization as well as heterodimerization between c-ErbB1 and c-ErbB2, c-ErbB3 and/or c-ErbB4, when present. Receptor dimerization leads to receptor autophosphorylation and activation of downstream signalling pathways including p42/p44 MAPK, JNK/SAPK, PI3K, PLC and cAMP/PKA (Dickson and Lippman 1995; Siegel *et al.*, 1998; Martinez-Lacaci *et al.*, 1999). TGFα is expressed in normal murine mammae within the basal cells of the epithelium and the terminal cells of the end buds (Snedeker *et al.*, 1991; Martinez-Lacaci *et al.*, 1999). It is also present in murine and human mammae during pregnancy (Liscia *et al.*, 1990) and has been demonstrated to have similar growth effects upon human and murine mammary epithelial cells *in vitro* (Salomon *et al.*, 1987; Bates *et al.*, 1988; Valverius *et al.*, 1989). Exogenous TGFα expression has also been reported to contribute to the transformation of murine MECs that have been previously immortalized, suggesting that growth factor expression can cooperate with other established genetic alterations in mammary tissue in transforming pathways (Shankar *et al.*, 1989; McGeady *et al.*, 1989). Early evidence demonstrated increased TGFα expression in mammary tumors versus normal mammary gland (Derynck *et al.*, 1987; Arteaga *et al.*, 1988; Bates *et al.*, 1988; Travers *et al.*, 1988); however, the current paradigm for EGF family growth factor participation in breast cancer also involves the establishment of a pro-survival, pro-proliferative, autocrine stimulatory loop with EGFR. The EGFR has also been found to be overexpressed with or without gene amplification in approximately 50% of breast cancers (Harris *et al.*, 1988; Dickson *et al.*, 1995; Dahiya *et al.*, 1998; Martinez-Lacaci *et al.*, 1999, De Luca *et al.*, 1999).

Three groups have independently developed transgenic mouse models in which the TGFα growth factor is expressed in a metal ion-inducible, general tissue context (MT1-*tgfα*) (Sandgren *et al.*, 1990; Jhappan *et al.*, 1990), a mammary-associated context (MMTV-*tgfα*) (Matsui *et al.*, 1990), or a mammary-specific context (WAP-*tgfα*) (Sandgren *et al.*, 1995). The two groups that generated MT1-*tgfα* transgenic mice used rat and human *tgfα* under the control of the heavy-metal inducible murine metallothionein (MT) promoter. Each group reported that TGFα expression significantly influenced mammary gland development and MEC proliferation as examined using mammary gland whole mounts. In addition, TGFα expression contributed to mammary alveolar hyperplasia and

mammary adenocarcinoma in multiparous female transgenic mice (Sandgren *et al.*, 1990; Jhappan *et al.*, 1990). MMTV-LTR-driven expression of the *tgfα* transgene was also shown to contribute to mammary alveolar hyperplasia in virgin female mice and to mammary adenocarcinoma in multiparous female mice. Furthermore, TGFα protein expression was confirmed and a TGFα/EGFR autocrine loop was suspected due to the increased presence of EGFR mRNA in areas of increased expression of the transgene (Matsui *et al.*, 1990). Finally, results from the characterization of the WAP-*tgfα* transgenic model suggest that constitutive *tgfα* expression accelerates mammary development, impedes apoptotic involution, and contributes to mammary transformation by acting as a survival factor for differentiated murine MECs (Sandgren *et al.*, 1995). Significantly, the requirement for pregnancy and the extended tumor latency for both TGFα transgenic models illustrated that TGFα is likely to be incapable of serving as the sole cause of mammary cancers. Rather, TGFα overexpression is likely to be one promotional step along a multistep oncogenic pathway(s). Therefore, it is particularly interesting that the tumorigenicity of cancer cell lines (liver) has been associated with the dual overexpression of *tgfα* and c-*myc*, suggesting a possible cooperativity between the two genes (Lee *et al.*, 1991).

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*MMTV-c-*myc*/MT-*tgfα *and WAP-c-*myc*/WAP-*tgfα *bitransgenic mice*

The MMTV-c-*myc*/MMTV-v-*Ha-ras* cross generated in 1987 was the first c-*myc*-containing bitransgenic mouse (Sinn *et al.*, 1987). Characterization of this bitransgenic mouse model demonstrated that deregulated c-*myc* expression synergized with deregulated v-*Ha-ras* expression to both accelerate mammary tumorigenesis and abrogate the requirement for pregnancy in this process. Interestingly, mammary tumors were demonstrated in both virgin female and male bitransgenic mice, despite a further delay in tumor onset in males of nearly 2 months. Eight years later, our group and another group reported the generation and characterization of mice bitransgenic for c-*myc* and *tgfα*, lending support to the notion that signalling through the EGFR and/or activation of Ras could synergize with deregulated c-*myc* expression in mammary tumorigenic processes (Amundadottir *et al.*, 1995; Sandgren *et al.*, 1995). The MMTV-c-*myc*/MT-*tgfα* bitransgenic mice from our laboratory develop multiple mammary adenocarcinomas with a much reduced latency, and do so in the absence of any requirement for pregnancy or ovarian hormone stimulation. These mammary adenocarcinomas grew without requirement for estrogen (i.e., without delayed tumor growth in ovariectomized bitransgenic female mice) despite being estrogen receptor positive, as shown by estrogen receptor ligand-binding assay. Furthermore, histological evaluation of mammary gland tissue from both female and male animals as young as 5 weeks of age evidenced both hyperplastic and neoplastic changes in areas of transgene co-expression (Amundadottir *et al.*, 1995). The complete absence of normal mammary tissue in bitransgenic animals and the ability of bitransgenic mammary tissue from 3 week-old mice to form tumors in athymic mice suggest that these two important, mammary gland-relevant genes (c-*myc* and *tgfα*) are capable of synergistically transforming the mammary epithelium, apparently requiring minimal, if any, additional genetic alterations (Amundadottir *et al.*, 1995,1996a). These studies also demonstrated that c-*myc* and *tgfα* are capable of further cooperation to drive hyperplastic and neoplastic changes in the murine salivary glands. This was not seen in single transgenic animals carrying c-*myc* or *tgfα* (Amundadottir *et al.*, 1995). Characterization of the WAP-c-*myc*/WAP-*tgfα* bitransgenic model confirmed the potent synergy of these two genes in promoting and accelerating mammary tumor formation, when compared with the relevant single transgenic animals. Furthermore, the power of this cooperative interaction between c-*myc* and *tgfα* is demonstrated in both our model and the WAP-based model since both male and virgin female bitransgenic animals develop mammary tumors (Amundadottir *et al.*, 1995; Sandgren *et al.*, 1995). The WAP promoter utilized in the latter study to drive the expression of the c-*myc* and *tgfα* transgenes is often presumed to drive transgene expression only in the latter part of pregnancy and throughout lactation and involution. However, the presence of mammary tumors in male and virgin female bitransgenics suggests that the MMTV and WAP promoters may be slightly 'leaky', in the sense that minimal transgene expression may still occur even in the absence of ovarian hormone stimulation → or that minimal promoter activity may be present during the estrous in these mice. (9)

Subsequent work in our laboratory with single transgenic mice, c-*myc*/*tgfα* bitransgenic mice, and cell lines derived from transgenic mouse mammary tumors, has led to the hypothesis that TGFα can cooperate with c-Myc in promoting cell cycle progression and can act to suppress c-Myc-induced apoptosis (Amundadottir *et al.*, 1996b; Nass *et al.*, 1996,1998). Our results, together with those from another group, have suggested that transformation, maintenance of transformation, and suppression of apoptosis in c-*myc*-overexpressing mammary tumor cell lines derived from transgenic animals may require signalling through the p42/p44 MAPK and PI3K pathways, both of which are targets of the activated EGFR (Amundadottir *et al.*, 1998; Wang *et al.*, 1999). *In situ* end labeling apoptosis assays (TUNEL staining) in paraffin-embedded mammary tumor sections from transgenic animals indicated the presence of apoptotic mammary cells in *c-myc* transgenic tumors and their near absence in tumors from the *tgfα* and c-*myc*/*tgfα* transgenic mice (Amundadottir *et al.*, 1996b). Data from our tumor cell lines indicate that coexpression of c-*myc* and *tgfα* results in increased cell proliferation under anchorage-dependent and anchorage-independent conditions, a reduced requirement for exogenous growth factor stimulation, and greatly decreased apoptosis. This protection from apoptosis is abrogated when EGFR signalling is blocked by addition of PD153035–a specific, synthetic EGFR tyrosine kinase inhibitor. Furthermore, the myc83 cell line, and an additional five other cell lines derived from mammary tumors in c-*myc* transgenic mice, were significantly more apoptotic than cell lines derived from *tgfα* and c-*myc*/*tgfα* bitransgenic mammary tumors. The frequency of apoptotic cells could be considerably suppressed by the addition of exogenous TGFα or EGF. Conversely, apoptosis was considerably accentuated when EGFR

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signalling was blocked via PD153035. This augmentation of apoptosis was sensitive to reversal by addition of the survival factor basic fibroblast growth factor (bFGF), which interacts with its own specific family of receptors and does not associate with EGFR (Amundadottir *et al.*, 1996b).

Molecular characterization of apoptosis in c-*myc*-overexpressing murine MECs derived from the MMTV-c-*myc* transgenic mice led to the recognition that Bcl-x_L, an anti-apoptotic member of the Bcl-2 family of apoptosis regulatory proteins, is a likely mediator of TGFα and EGF-directed protection against c-*myc*-driven apoptosis. Bcl-x_L mRNA and protein levels were elevated with TGFα or EGF treatment of these c-*myc*-expressing cell lines, and expression of this anti-apoptotic molecule was significantly diminished with growth factor removal, TGFβ treatment, or PD153035 treatment. In addition, levels of Bax (a pro-apoptotic Bcl-2 family member) and p53 appeared relatively high and unchanged, while Bcl-2 and Bcl-x_S (another pro-apoptotic Bcl-2 family member) levels remained low or undetectable with these aforementioned treatments (Nass *et al.*, 1996). The work in our laboratory has led to the following models for the cooperation between c-Myc and TGFα in proliferation and apoptosis in the mouse mammary gland: First, with respect to proliferation, deregulated c-Myc may drive cellular proliferation by upregulating cyclin E, cdc25A phosphatase, and CAK-activating partner cdk7, and by lowering p27 levels resulting in cdk2 activation. In contrast, TGFα overexpression leads to the induction of cyclin D1 and, subsequently, the activation of cdk4/6. The combination of these two effects may further deregulate the cell cycle. Second, with respect to apoptosis, deregulated c-*myc* expression may promote apoptosis by directly inducing p53 expression, and by directly or indirectly inducing Bax expression. Bax has been shown to be directly responsive to p53 and also to be a potential target for c-Myc induction because four E-boxes are located in the Bax promoter/5'-UTR (Miyashita *et al.*, 1995). At present, there is no published evidence that c-Myc functions through these elements to induce Bax expression. As previously mentioned, TGFα appears to activate cellular survival pathways and induce the expression of the anti-apoptotic protein Bcl-x_L (Nass *et al.*, 1996). This work, combined with results from the characterization of *MMTV*-c-*myc*/WAP-*bcl*-2 bitransgenic mice, strongly suggests that mammary tumorigenesis is significantly increased when deregulated c-*myc* expression, responsible both for driving cellular proliferation as well as increasing cellular sensitivity to apoptosis, is coupled with other genetic alterations that act as survival signals to block c-*myc*-mediated apoptotic pathways. In this latter study, *bcl-2* expression accelerated mammary tumorigenesis and suppressed *in vivo* mammary tumor apoptosis (Jäger *et al.*, 1997).

p53 *tumor suppressor gene, the mammae and breast cancer*

p53 is a 393-amino acid nuclear phosphoprotein transcription factor known to bind DNA upon stabilization induced by cell cycle checkpoint controls. p53 transactivation increases the expression of genes involved in such distinct processes as apoptosis, DNA repair, and cell cycle arrest (Evan *et al.*, 1998; El-Deiry, 1998). p53 has often been termed the 'guardian of the genome' owing to the fact that it plays such a critical role as a tumor suppressor by orchestrating cell cycle and DNA repair upon recognition of certain levels of DNA damage. Cell cycle inhibitory activities are believed to be controlled by p53-dependent transcriptional activation of genes, including p21/WAF1/CIP1, 14-3-3σ, and GADD45. In addition to its role in DNA damage recognition, the p53 tumor suppressor has also been linked to the recognition of oncogene activation (c-*myc* and adenovirus E1A), subsequently resulting in apoptosis induction via a pathway that includes ARF and MDM2 (Zindy *et al.*, 1998; de Stanchina, *et al.*, 1998; Sherr, 1998). p53 is capable of promoting apoptosis upon recognition of severe, irreparable DNA damage, DNA damage in the context of other environmental conditions unfavorable for maintenance of genomic integrity, and abnormal cellular proliferation as driven by oncogene activation. Thus far, p53-dependent apoptosis has been demonstrated to result from the transcriptional activation of genes, including Bax, Fas/Apo1/CD95, and DR5 Trail receptor, and from transcriptional repression of the anti-apoptotic gene Bcl-2 (Canman *et al.*, 1997; El-Deiry, 1998).

Little information exists concerning the expression pattern for wild-type p53 during development in either human or murine mammary glands. One study indicates that p53 mRNA is expressed during pregnancy and involution, but not during lactation (Strange *et al.*, 1992). Another study, however, suggests that the complete absence of p53 expression does not alter the histological or functional development of the mammae in mice, since *p53*$^{-/-}$ mice remain capable of lactation (Donehower *et al.*, 1992). As regards the role of p53 in mammary apoptosis, both p53-dependent and p53-independent apoptosis have been demonstrated in cultured MECs (Merlo *et al.*, 1995). In mice, one study has indicated that post-lactational mammary involution and apoptosis proceed normally without regard for p53 status (Li *et al.*, 1996); whereas, another study has demonstrated that the first phase of mammary involution is delayed in p53-null animals (Jerry *et al.*, 1998).

The *p53* tumor suppressor is one of the most frequently altered genes in a wide variety of human cancers, including breast cancer (Donehower *et al.*, 1993). Breast cancer, along with sarcomas, brain tumors, leukemias and adrenal cortical tumors, is common among women with Li-Fraumeni Syndrome, a disorder linked to germline mutations in the *p53* locus (Eeles *et al.*, 1993). Furthermore, *p53* gene mutations have been identified in approximately 17% of all human breast cancers (Dahiya and Deng, 1998). To date, results in the mammary glands of murine *p53*-knockout animals have been somewhat discordant with expectations based on Li-Fraumeni Syndrome. Specifically, non-mammary gland tumors, such as lymphomas, rapidly arise in *p53*-knockout animals, suggesting that p53 is not of predominant importance in murine mammary tumor development (Donehower *et al.*, 1992; Harvey *et al.*, 1993; Purdie *et al.*, 1994). More recent investigations of human breast cancer-relevant *p53* missense mutations expressed in transgenic models (Li *et al.*, 1998) and *wnt1* transgenic/*p53*-knockout murine

models (Donehower *et al.*, 1995; Jones *et al.*, 1997) indicate that p53 alteration can be contributory to mammary tumorigenesis in some circumstances. It is possible that the lack of agreement concerning the role of p53 loss in murine models of cancer and human breast cancer results from interspecies differences, from the modulation of tumorigenesis by murine strain differences, from other transgenes carried in the background, and from the particular *p53* genetic knockouts and mutations modeled in these mice. The latter difference may be most significant, since the mammary tumorigenic effects noted in the study of the p53-172R/H mutant transgenic mouse resulted from the rational modeling of a specific, human breast cancer-relevant p53 alteration (Li *et al.*, 1998).

MMTV-c-myc/p53$^{+/-}$ *transgenic mice*

In 1995, two transgenic models with a mammary-targeted oncogene (MMTV-*wnt1* or MMTV-c-*myc*) and *p53* deficiency were established to determine whether or not deficiencies in the tumor suppressor *p53* could cooperate with deregulated expression of Wnt1 or c-Myc to alter tumorigenesis in mammary tissues (Donehower *et al.*, 1995; Elson *et al.*, 1995). A cooperative effect was indeed observed between Wnt1 and *p53* deficiency, as mammary tumors in the MMTV-*wnt1*/*p53*$^{-/-}$ mice arose sooner and had a significantly higher degree of chromosomal instability than those of MMTV-*wnt1*/*p53*$^{+/-}$ and MMTV-*wnt1*/*p53*$^{+/+}$ animals (Donehower *et al.*, 1995). In the MMTV-c-*myc* model, animals with *p53* disruption rapidly developed lethal lymphomas, indicating that c-*myc* and mutant p53 had a cooperative effect in terms of increasing the incidence and accelerating the onset of T-cell lymphomas. However, *p53* disruption failed to influence the mammary adenocarcinoma phenotype of the MMTV-c-*myc* animals. In those *MMTV-c-myc/p53*$^{+/-}$ female mice that survived their lymphomas long enough to acquire mammary tumors, there was no identifiable alteration in tumor latency, histology, or dependence upon pregnancy as compared with MMTV-c-*myc*/*p53*$^{+/+}$ controls (Elson *et al.*, 1995). The absence of cooperation between p53 and c-Myc in terms of mammary carcinogenesis in this model may reflect intrinsic differences between murine and human mammary tumorigenesis, the cooperation between c-*myc* and *p53* in inducing extremely aggressive lymphomas that limited the mammary observation window, or the specific manner in which the *p53* alleles were targeted. It has been demonstrated that most *p53* alterations in human breast cancers are missense mutations that may influence the activity of the *p53* gene product, rather than deletions of entire *p53* exons (as was done in both of the previously mentioned models) that are capable of completely eliminating all p53 functionality (Elson *et al.*, 1995; Lozano *et al.*, 1998). This particular fact suggests that a cross between the p53-172R/H mutant transgenic mouse and WAP-c-*myc* or MMTV-c-*myc* transgenic mouse might be more relevant to the study of breast cancer. Unfortunately, there is no evidence to date concerning the frequency or relevance of combined c-*myc* amplification/overexpression and *p53* mutation in human breast tumors.

Recently, our group generated transgenic mice in which the mammary-targeted c-*myc* oncogene was expressed in the presence of a targeted disruption of the *p53* tumor suppressor gene (McCormack *et al.*, 1998). Although our results indicated that disruption of *p53* may contribute to alveolar hyperplastic changes in the virgin female transgenic mouse, they failed to show any cooperation between c-*myc* and *p53* disruption in mammary tumorigenesis, since no alterations in latency, histology, or apoptosis were observed between c-*myc*-induced mammary tumors in animals with or without disrupted *p53* (McCormack *et al.*, 1998). To determine whether or not disruption of *p53* could influence c-*myc*-induced chromosomal instability in mammary tumors from these transgenic mice, tumor-derived cell lines were subjected to spectral karyotyping (SKY) analysis (Liyanage *et al.*, 1996). This analysis demonstrated that *p53* disruption did not significantly influence ploidy or other c-*myc*-induced chromosomal alterations. Analysis of these *p53*$^{-/-}$ and *p53*$^{+/-}$ tumor cells lines using both SKY and comparative genomic hybridization (CGH) also supported the concept that c-*myc*-induced chromosomal instability is unaffected by *p53* status (McCormack *et al.*, 1998; Weaver *et al.*, 1999). Unfortunately, the effects of complete *p53* disruption in the presence of c-*myc* transgene expression were untestable due to rapidly arising lymphomas that forced us to limit the time for mammary observations.

Summary and future directions

Recent progress in the study of c-Myc has convincingly demonstrated that it possesses a dual role in promoting cellular proliferation and apoptosis. Work from our group and others has confirmed this dual role of c-Myc in murine mammae and has further shown that co-expression of TGFα can synergistically accelerate mammary tumorigenesis as well as abrogate tumor reliance on estrogenic signalling. These results appear similar to those obtained for c-*myc*/v-*Ha-ras* bitransgenic mice and further suggest that signalling through the EGFR (as well as activation of Ras) may induce downstream survival-signalling pathways that impinge upon c-Myc-driven apoptosis. Currently, work is being conducted in our laboratory with mammary tumor cell lines derived from the bitransgenic mice to resolve the nature and contribution of these survival pathways. The contribution of p53 mutation to breast tumorigenesis in humans is well established. Nevertheless, several studies suggest that p53 loss does not functionally or physically alter the murine mammae. Work from our group and another group has indicated a lack of obvious cooperation between hemizygous p53 knockout and c-*myc* transgene expression in bitransgenic mice. Unfortunately, the nature of these two models precluded the examination of the effect of homozygous p53 loss on mammary tumorigenesis due to the pervasive and aggressive lymphomas that arose in these animals. As was suggested by work with the p53-172R/H mutant mouse, it would be worth examining the contribution of breast cancer-specific p53 point mutants to c-*myc*-induced mammary tumorigenesis.

Of significant interest to those who study breast cancer and c-Myc is the nature of apoptosis signalling by c-Myc and its contribution to breast tumorigenesis.

Greater resolution of this apoptotic pathway could suggest additional targets for breast cancer therapies. Work described herein provides the basis for the development of other combinatorial, mammary-specific transgenic models that will further dissect the relationship between c-Myc and apoptosis.

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